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In re PCT National Stage Application of:

Glue, *et al.*

U.S. Application No.: 10/576,052

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Filed: 26 October 2004

For: **Use Of Neurokinin Antagonists In The Treatment Of Urinary Incontinence**

Confirmation No.: 6943

Art Unit: 1614

Examiner: Spivack, Phyllis G.

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DECLARATION OF ECKHARD WEBER UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Eckhard Weber, do declare that:

1. I am a co-inventor of the inventions disclosed and claimed in US Patent Application Serial No. 10/576,052 (the "'052 Application").

2. I am a PhD scientist and am currently working in the Gastrointestinal Disease Area at Novartis Pharma AG in Basel, Switzerland. I have more than 15 years of experience in pre-clinical research, which includes about two years of pre-clinical research involving urinary incontinence.

3. I have reviewed the office action dated 21 March 2008 in which the Examiner rejected Claims 1-8 under 35 U.S.C. § 103(a) as being unpatentable over U.S. 2004/0058914 (the "Doi reference"). I have also reviewed the Doi reference. In the Office Action the Examiner states that the Doi reference teaches the administration

of the neurokinin receptor antagonist DNK333 (the compound of Claim 7 in the '052 Application) in the treatment of urinary incontinence.

4. However, the Doi reference does not demonstrate that any of the compounds disclosed in the Doi reference are effective to treat urinary incontinence. The only experimental data in Doi that purport to demonstrate the effectiveness of the disclosed compounds in treating urinary incontinence are in Experimental Examples 1 and 2; however, that experimental data instead demonstrates the effectiveness of a combination of disclosed compounds in increasing cyclophosphamide-impaired bladder capacity. The model used in Experimental Examples 1 and 2 that purportedly tested for the efficacy of a composition with respect to urinary incontinence but which instead tested for the efficacy of a composition with respect to increasing bladder capacity was based on the treatment of animals under urethane anesthesia. It is unclear whether the experiments were performed in rats (as described in the text of Experimental Examples 1 and 2) (see paragraphs [0501] and [0503] of the Doi reference) or in guinea pigs (as described in Tables 1 and 2 in Experimental Examples 1 and 2). Since the text refers multiple times to rats, it is believed that the model used in the Doi reference was a rat model, as opposed to a guinea pig model. Species differences regarding their neurokinin receptor homology are well known. Data generated in rat models are considered to be of low predictability for humans because of the low homology of rat and human NK_1 receptors, (See Beresford et al., "Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist, CP-96,345, " British Journal of Pharmacology, Vol. 104, No. 2, pp 292-293 (1991) (a copy of which is attached).

5. As is well known, clyclophosphamide is a chemotherapy agent that is used in the treatment of lymphomas. As set forth in Alfieri, et. al., "Nitric Oxide and NK_1-Tachykinin Receptors in Cyclophosphamide-Induced Cystitis, in Rats," Pharmacology and Experimental Therapeutics, Vol. 295, No. 2, pp 824-829 (2000) (a copy of which is attached), cyclophosphamide is known to induce cystitis in rats. The Alfieri article discloses that cyclophosphamide-induced cystitis in rats can be ameliorated by NK_1

antagonists. The Alfieri article demonstrates that the rat model used by Doi is a model for cystitis. [1]

6. Since cystitis is an inflammatory condition, the model used in the Doi reference would generate results with respect to the treatment of an inflammatory condition and would not indicate whether the compounds effective in treating cystitis would be effective in treating urinary incontinence. As set forth in Karl Erik Andersson, "Tachykinins: Role in Detrusor Overactivity?" European Urology, Vol. 49, pp. 423-425 (2006) (a copy of which is attached), there may be different mechanisms for various bladder disorders. Urinary incontinence is generally attributed to sphincter incompetence or detrusor muscle overactivity, both of which involve a neuromuscular mechanism, and if inflammation plays a role in urinary incontinence, it is a minor role. Both sphincter incompetence or detrusor muscle overactivity involve a neuromuscular mechanism for urinary incontinence. However, the model used in the Doi reference is a model that is not directed towards a neuromuscular mechanism for urinary incontinence and is not a model for urinary incontinence.

7. The Experimental Examples in Doi demonstrate that an NK_1 receptor antagonist alone is not effective in treating the condition present in the model employed. In Green, et al., "Efficacy and Safety of a Neurokinin-1 Receptor Antagonist in Postmenopausal Women with Overactive Bladder with Urge Urinary Incontinence," The Journal of Urology, Vol. 176, pp. 2535-2540 (2006) (a copy of which is attached), the results from a clinical trial where an NK_1 receptor antagonist alone was used to treat urinary urge incontinence are presented. The Green article states that the clinical trial demonstrated the efficacy for an NK_1 receptor antagonist in urge urinary incontinence (see page 2538). Since the Experimental Examples in Doi demonstate that an NK_1 receptor antagonist alone is not effective in treating the condition being treated in the

[1] As noted in paragraph 4, it is unclear whether rats or guinea pigs were used in Experimental Examples 1 and 2 but it is believed that rats were used since the text in Experimental Examples 1 and 2 refers multiple times to rats. Regardless of whether rats or guinea pigs were used, cyclophosphamide was used in both Experimental Examples 1 and 2 and would have induced cystitis in the animals that were used, and therefore, the model that was used in Experimental Examples 1 and 2 was a model of cystitis.

Doi model whereas the Green article demonstrates that an NK_1 receptor antagonist alone is effective in treating urinary incontinence, it further demonstrates that the Doi reference is using a model that involves a condition other than urinary incontinence and is not a model for urinary incontinence.

8. In short, the model used in the Doi reference is a model for cystitis, which is an inflammatory condition. Since inflammation plays a minor role, if any, in urinary incontinence, the Doi reference has not demonstrated that any of its compounds are useful in treating urinary incontinence.

9. In contrast to the model used in the Doi reference, an in vivo model of stimulated micturition in conscious guinea pigs and an isolated guinea pig detrusor contractility model were used in the '052 Application (see Specification, p. 10, paragraph 2). Guinea pig models were used because of the high homology of guinea pig and human NK receptors. The in vivo model of stimulated micturition (see Example 1 of the Specification) is based on the subcutaneous administration of 5-hydroxytryptophan (5-HTP), which is a precursor for serotonin, which is a key neurotransmitter of the viscera and triggers neuromuscular detrusor contractions. The other model (see Example 2 of the Specification) is based on the application of substance P, which is the endogenous ligand for NK receptors and also a key neurotransmitter triggering neuromuscular detrusor contractions. Both models used in the '052 Application tested DNK333, which is a dual NK_1 and NK_2 antagonist, focused on neuromuscular mechanisms for urinary incontinence, such as a neuromuscular mechanism of detrusor overactivity, and are models for non-inflammatory overactive bladder/urinary incontinence. Therefore, the experimental results in the '052 application demonstrate preclinical efficacy of the compounds of formula I of Claim 1 in the '052 Application for the treatment of urinary incontinence.

10. The Doi reference states:

> The present invention aims at providing a pharmaceutical agent that can be widely applied to diseases such as urinary frequency, urinary incontinence, asthma, chronic obstructive pulmonary disease,

rheumatoid arthritis, osteoarthritis, pain, cough, irritable bowel syndrome, emesis, depression, anxiety, manic depression psychosis, schizophrenia and the like. (See paragraph [0009].)

As shown above, the Doi reference has not provided any experimental evidence that the compounds disclosed in the Doi reference are effective in treating urinary incontinence. It has only provided experimental evidence with respect to the treatment of cystitis. Further it has only provided experimental evidence with respect to combination treatment. As a result, based on the experimental data provided by the Doi reference, one of ordinary skill in the art could not conclude from such experimental data that a compound according to formula I of Claim 1 in the '052 Application would be effective in the treatment of urinary incontinence.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Sept 17th, 2008

Eckhard Weber

Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist, CP-96,345

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The affinity of the non-peptide antagonist CP-96,345 for tachykinin NK_1 receptors has been estimated in a range of species by use of both radioligand binding and functional assays. CP-96,345 was 30–120 fold less active at NK_1 receptors in rat and mouse than in the other species examined, including man. These results demonstrate the existence of species variations in NK_1 receptors.

Keywords: Tachykinin NK_1 receptor; NK_1 receptor antagonist; species variants; subtype, CP-96,345

Introduction Receptors for the mammalian tachykinins, substance P (SP), neurokinin A and neurokinin B, have been classified on pharmacological criteria into three subtypes, termed NK_1, NK_2 and NK_3 (see review by Guard & Watson, 1991). This classification has been substantiated by the cloning of all three receptors (Nakanishi, 1991). However, functional studies *in vitro* using early peptide antagonists suggested that subtypes of the NK_1 receptor may exist (Brown *et al.*, 1985a,b) and, recently, in NK_1 receptor binding assays, the affinity of the non-peptide tachykinin NK_1 receptor antagonist CP-96,345 has been shown to be species-dependent (Gitter *et al.*, 1991; Snider *et al.*, 1991).

To investigate further the possible existence of species subtypes of the NK_1 receptor, we have measured the ability of racemic CP-96,345 to inhibit binding of [^{3}H]-SP to brain cortex membranes prepared from eight different species and have determined whether estimates of affinity at [^{3}H]-SP binding sites correlate with those determined at NK_1 receptors using functional assays *in vitro*.

Methods *[^{3}H]-substance P binding* [^{3}H]-SP binding assays were performed essentially as described by Dam & Quirion (1986). Cerebral cortical membranes (8–15 mg wet weight per assay tube) were incubated with [^{3}H]-SP (0.5–0.7 nM, specific activity 34 Ci mmol^{-1}, DuPont) at 22°C for 40 min. Non-specific binding was defined as that remaining in the presence of physalaemin (1 μM).

Smooth muscle preparations Rings of rabbit thoracic aorta (male New Zealand White rabbits, 2–3 kg, Froxfield) or sections of guinea-pig ileum longitudinal smooth muscle (male Dunkin-Hartley guinea-pigs, 300–500 g, Porcellus) were prepared as described by Regoli *et al.* (1984). Preparations were mounted in organ baths (37°C) filled with either Krebs-Henseleit medium containing indomethacin (1 μM) (aorta) or Tyrode solution containing atropine, indomethacin, mepyramine, methysergide and ondansetron, all at 1 μM (ileum). Mechanical activity was recorded isometrically.

Neonatal rat spinal cord Spinal cord was excised from C.D. rat pups (1–8 days *post partum*, Glaxo), hemisected sagitally and superfused (2 ml min^{-1}) with modified Krebs-Henseleit medium (containing $MgSO_4$ 0.7 mM and $CaCl_2$ 1.2 mM) at room temperature. Depolarization responses were recorded extracellularly from lumbar (L3–L5) ventral roots (see Brown *et al.*, 1985a).

Experimental design Aorta preparations were pre-contracted with phenylephrine (0.1 μM). Concentration-relaxation response curves to substance P methylester (SPOMe) were constructed by cumulative addition. For ileum and spinal cord, concentration-response curves to SPOMe were constructed non-cumulatively by use of serially-increasing concentrations. Experiments to determine the apparent affinity of antagonists were undertaken as described previously (see Ireland *et al.*, 1991).

Data analysis Binding data were analysed by the curve-fitting programmes ALLFIT and LIGAND. From functional assays, the apparent affinity of antagonist (pK_B) was estimated as described previously (Ireland *et al.*, 1991).

Drugs Racemic CP-96,345 [*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine] was synthesized in the Department of Medicinal Chemistry, Glaxo Group Research, Ware, Herts. Physalaemin and SPOMe were supplied by Peninsula and Cambridge Research Biochemicals, respectively.

Results *[^{3}H]-substance P binding* CP-96,345 potently inhibited binding of [^{3}H]-SP to rabbit, guinea-pig, human, bovine, hamster and gerbil cerebral cortices with similar nanomolar potencies (Table 1). In contrast, CP-96,345 was 30–120 fold less potent in rat and mouse tissues (Table 1). In comparison, the NK_1 agonist physalaemin was equipotent in all species (Table 1). Saturation analysis of [^{3}H]-SP binding (0.02–10 nM) to rabbit, guinea-pig and rat cerebral cortex indicated that [^{3}H]-SP bound to single populations of binding sites with equilibrium dissociation constants (K_D) of 165 ± 2, 122 ± 2 and 106 ± 24 pM, respectively ($n = 4$). Maximal

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Table 1 Comparison of the potencies (pIC_{50}) of CP-96,345 and physalaemin to inhibit binding of [^{3}H]-substance P ([^{3}H]-SP) to cerebral cortical membranes prepared from different species

	Rabbit	*Guinea-pig*	*Man*	*Bovine*	*Hamster*	*Gerbil*	*Rat*	*Mouse*
CP-96,345	8.62 ± 0.06	8.50 ± 0.08	8.46	8.86 ± 0.12	8.40 ± 0.01	8.51 ± 0.11	6.77 ± 0.08	6.92 ± 0.08
Physalaemin	8.13 ± 0.05	7.92 ± 0.10	n.d.	8.10 ± 0.01	8.35 ± 0.06	7.99 ± 0.03	8.12 ± 0.08	8.25 ± 0.09

Results are mean pIC_{50} values ± s.e.mean of 3–10 experiments, except man ($n = 1$). Slopes of displacement curves were not significantly different from unity.

binding capacities were calculated to be 103 ± 11, 25 ± 1 and 64 ± 10 fmol mg^{-1} protein ($n = 4$) in rabbit, guinea-pig and rat cortex, respectively. Using the K_D determinations, pKi values for CP-96,345 were calculated to be 9.30 ± 0.08 ($n = 10$), 9.18 ± 0.12 ($n = 5$) and 7.65 ± 0.06 ($n = 5$) in rabbit, guinea-pig and rat, respectively.

Functional responses CP-96,345 behaved as a reversible competitive antagonist of responses induced by SPOMe in the rabbit aorta, guinea-pig ileum and neonatal rat spinal cord. Thus, in the presence of CP-96,345, concentration-response curves were displaced to the right in a concentration-dependent and parallel manner (Figure 1). Further, Schild plots constructed from the antagonism data had gradients not significantly different from unity. The estimated values were 0.89 (95% confidence limits 0.66–1.11, $n = 20$), 0.78 (0.40–1.16, $n = 15$) and 0.94 (0.80–1.07, $n = 9$) in aorta, spinal cord and ileum, respectively (Figure 1). The apparent affinity of CP-96,345 was similar in the rabbit aorta and guinea-pig ileum (pK_B 8.81 ± 0.06 ($n = 20$) and 8.89 ± 0.02 ($n = 9$), respectively). In contrast, CP-96,345 was markedly less potent in the neonatal rat spinal cord (pK_B 7.13 ± 0.10 ($n = 15$)). CP-96,345 (100 nM) had no effect on contractions induced by either carbachol or bradykinin in guinea-pig ileum or by phenylephrine in rabbit aorta (data not shown).

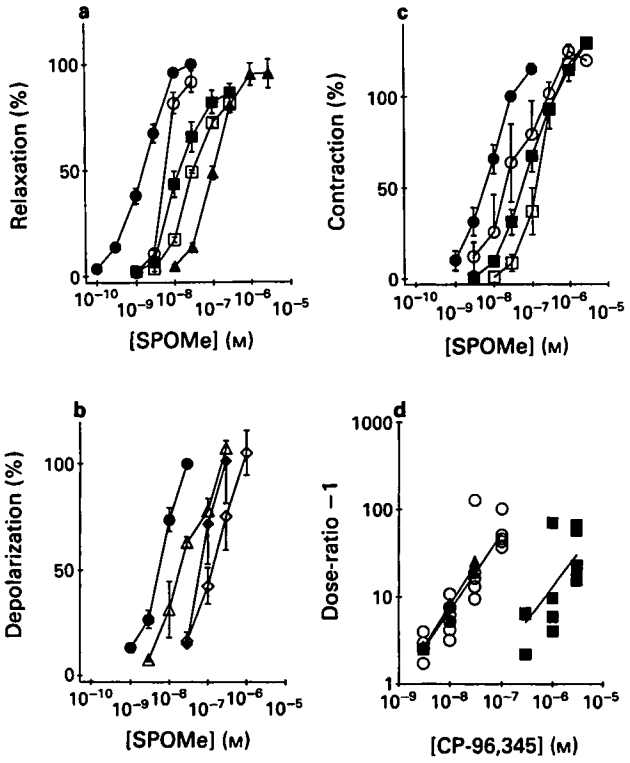

Figure 1 Antagonism by CP-96,345 of responses to substance P methyl ester (SPOMe) in the rabbit thoracic aorta (a), neonatal rat spinal cord (b), or guinea-pig ileum (c). Data are expressed as a mean percentage of the response to SPOMe (30 nM). Symbols indicate controls (●), or the presence of CP-96,345 at 3 (○); 10 (■); 30 (□); 100 (▲); 300 (△); 1000 (◆) or 3000 (◇) nM. Each point is mean of single determinations in at least 3 separate preparations; vertical bars show s.e.mean. (d) Schild plots for CP-96,345 antagonism of SPOMe-induced responses in rabbit thoracic aorta (○), guinea-pig ileum (▲) or neonatal rat spinal cord (■). Data were derived from the experiments illustrated in Figure 1a–c. Each point represents data obtained from a separate preparation.

Discussion The observation that the non-peptide NK_1 receptor antagonist, CP-96,345 was approximately 30–120 fold less potent at inhibiting [^{3}H]-SP binding in rat or mouse cerebral cortex than other mammalian species (including man) is in agreement with previous studies suggesting that this compound can discriminate species variants of NK_1 receptors (Gitter *et al.*, 1991; Snider *et al.*, 1991). These differences are unlikely to arise from differential affinities of [^{3}H]-SP for cortical binding sites since the K_D value for the ligand was very similar in rat, rabbit and guinea-pig cortex. In addition, the NK_1 agonist, physalaemin, was equipotent in all species tested.

Importantly, the observed differences in binding affinities were reflected in antagonist potencies at functional NK_1 receptors in representative isolated preparations. Thus, there was good agreement between estimates of apparent affinity in functional preparations and binding studies conducted in tissue from the same species: in rat spinal cord and rat cortex, CP-96,345 was approximately 40 fold weaker than in rabbit aorta and rabbit cortex or guinea-pig ileum and guinea-pig cortex.

The present results demonstrate that the affinity of CP-96,345 for functional NK_1 receptors is species-dependent. They are also consistent with the suggestion that NK_1 receptors can be resolved into two groups, those in rabbit, guinea-pig, human, cow, hamster and gerbil being distinct from those in rat and mouse. The possibility of further subdivision of the NK_1 receptor either between or within species remains to be addressed.

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Nitric Oxide and NK_1-Tachykinin Receptors in Cyclophosphamide-Induced Cystitis, in Rats[1]

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ABSTRACT

The present study was conducted to investigate the role of NK_1 receptors and of nitric oxide (NO) on the pathogenesis of cyclophosphamide-induced cystitis, in rats. This bladder toxicity was characterized by marked increases in protein plasma extravasation, urothelial damage, edema, white blood cell infiltrates, and vascular congestion. These changes were associated with appearance of Ca^{2+}-independent NO-synthase (NOS) activity [characteristic of inducible NOS (iNOS)] in the bladder and with increases in urinary NO metabolites. GR205171, a selective NK_1 antagonist (10–20 mg/kg, i.p.) reduced cyclophosphamide-induced increases in protein plasma extravasation and in the urinary excretion of NO metabolites. N^G-Nitro-L-arginine (L-NNA) (10 mg/kg, i.p.), a NOS inhibitor, reduced basal and cyclophosphamide-induced increases in NO metabolites and protected against cyclophosphamide-induced protein plasma extravasation. GR205171 had no effect, whereas L-NNA reduced basal NO metabolite excretion. Combined treatment with the NK_1 antagonist and the NO-synthesis inhibitor produced comparable reduction in protein plasma extravasation than that achieved with each drug given separately. Combined drug treatment ameliorated cyclophosphamide-induced urothelial damage, and the extent of edema, vascular congestion, and white blood cell infiltrates in the bladder. In summary, NK_1 receptors and iNOS play a role in NO formation and on cyclophosphamide-induced cystitis. Activation of NK_1 receptors mainly acts through the formation of NO. It is proposed that cyclophosphamide and/or its metabolites would stimulate primary afferent capsaicin-sensitive fibers in the bladder, releasing neuropeptides, which would activate NK_1 receptors. However, additional mechanisms are involved, because neither the NK_1 receptor antagonist nor the NO synthesis inhibitor, either alone or in combination, were able to completely prevent the toxicity.

Severe cystitis has been reported in laboratory animals after cyclophosphamide (CYP) administration (Ahluwalia et al., 1994; Alfieri and Gardner, 1997) and in patients receiving the drug as part of their treatment (Frasier et al., 1991). CYP is a drug with a wide spectrum of clinical uses, and it has been proved to be effective in the treatment of cancer and nonmalignant disease states. However, unless precautions are taken, this drug may induce acute inflammation of the urinary bladder (Grinberg-Funes et al., 1990). The genesis of this inflammation is being examined.

Pretreatment with the tachykinin NK_1 receptor antagonist, GR203040, has been shown to reduce the magnitude of CYP-induced cystitis (Alfieri and Cubeddu, 1997; Alfieri and Gardner, 1997). Other investigators have also shown that primary afferent capsaicin-sensitive fibers (PACSF), through the release of substance P (sP), neurokinin A, and/or calcitonin gene-related peptide, play an important role in animal models of cystitis (Maggi et al., 1987; Chahl, 1988). However, the mechanism by which NK_1 receptor inhibition protects against CYP-induced cystitis, is unclear.

Nitric oxide (NO) synthesis is mediated by three different types of nitric-oxide synthases (NOS): neuronal, endothelial, and inducible (Moncada et al., 1991). The first two synthases are expressed constitutively and are calcium-dependent, whereas inducible NOS (iNOS) must be induced and is calcium-independent (Moncada et al., 1991). It is well accepted that NK_1 receptor activation can induce the synthesis and release of NO (Regoli et al., 1994; Maggi, 1997). In addition, different agents (chemical, biological, and physical) could also trigger the inflammatory signal via transcriptional factors like NF-κB, or immune-related mediators such as interleukins, tumor necrosis factor α, and platelet-activating factor (Pfeilschnifter et al., 1992; Souza-Filho et al., 1997). These factors are known to increase the levels of iNOS, producing large amounts of NO, vasodilation, and edema.

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ABBREVIATIONS: CYP, cyclophosphamide; NK_1, neurokinin-1; NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; PACSF, primary afferent capsaicin-sensitive nerve fibers; L-NNA, N^G-nitro-L-arginine; GR205171, (2S,3S)-2-methoxy-(5-trifluoromethyltetrazol-1-yl-benzyl)-(2-phenylpiperidin-3-yl)amine hydrochloride; sP, substance P.

Based on these observations, we propose that CYP (and/or its metabolite acrolein) may stimulate PACSF to release sP and related substances, which through activation of NK_1 receptors my increase NO production inducing inflammation and damage. We propose that the reported amelioration of CYP-induced inflammatory cystitis with NK_1 antagonists is due to a reduction in the formation of NO. To evaluate this hypothesis, first we evaluated the role of NO on CYP-induced cystitis. Bladder iNOS was determined by measuring calcium-independent NOS activity in control and in animals treated with CYP. The urinary excretion of NO metabolites was used as an indicator of NO production. The effects of a NO synthesis inhibitor on NO production, protein plasma extravasation, and bladder toxicity were evaluated. Second, we investigated whether increases in NO formation mediate the protective effect of the NK_1-receptor antagonist (GR205171) on CYP-induced cystitis. To determine the relative contributions of NOS and of NK_1 receptors to the toxicity, the effects of separate and of combined treatments with these agents were evaluated.

Materials and Methods

Wistar male rats (body weight, 300–400 g) were used in all experiments. When administered, CYP was injected i.p. at a dose of 150 mg/kg. GR205171 [(2*S*,3*S*)-2-methoxy-(5-trifluoromethyltetrazol-1-yl-benzyl)-(2-phenylpiperidin-3-yl)amine hydrochloride] was used as the selective NK_1 antagonist. NO synthase was inhibited by the use of *S*(+)-N^5-[imino(nitroamino)methyl]ornithine (N^G-nitro-L-arginine; L-NNA).

The animals were included in one of eight groups: group 1, control (saline, 0.1 ml/100 g, i.p.); group 2, GR205171 (10 mg/kg, i.p.); group 3, L-NNA (10 mg/kg, i.p.); group 4, CYP + saline (0.1 ml/100 g, i.p., 5 min before and 3 h after CYP); group 5, CYP + GR205171 (10 mg/kg, i.p., 5 min before CYP); group 6, CYP + GR205171 (10 mg/kg × two doses, 5 min before and 3 h after CYP); group 7, CYP + L-NNA (10 mg/kg, i.p., 5 min before CYP); and group 8, CYP + L-NNA (10 mg/kg, i.p., 5 min before) + GR205171 (10 mg/kg × two doses, 5 min before and 3 h after CYP).

Plasma Protein Extravasation. Plasma protein extravasation was measured by the Evans blue dye leakage technique (Saria and Lundberg, 1983). Anesthesia was induced by the i.p. administration of urethane (1.2 g/kg). An external jugular vein was cannulated for the injection of Evans blue dye (50 mg/kg) in a dose volume of 2.5 ml/kg. The dye was administered 15 min before the animal was exsanguinated by infusion of 50 ml of 0.9% w/v saline, at 37°C, into the left cardiac ventricle. The time of exsanguination was taken as the endpoint of the experiment. After this, the urinary bladder was removed and blotted dry before weighing, and the content of dye was determined by spectrophotometry (at 620 nm), after extraction in a known volume of formamide at 60°C for 24 h. Plasma protein extravasation was expressed as the content of Evans blue dye in micrograms per gram of tissue.

Histological Study. Histological examination of the bladder was performed in three groups of animals. Controls (group 1), CYP (group 4), and CYP + L-NNA (one dose of 10 mg/kg) and + GR205171 (two doses of 10 mg/kg each) (group 8). None of these animals received the Evans blue dye. The tissue samples were fixed overnight in buffered neutral formalin, processed to paraffin wax, sectioned at 3 to 4 μm, and stained with hematoxylin and eosin. Extents of white blood cell infiltrates were graded in a 10× field from 0 to 4 as follows: 0, no extravascular leukocytes; +, ≤10 leukocytes; ++, 11 through 19 leukocytes; +++, 20 through 29 leukocytes; and ++++, ≥30 leukocytes.

Urinary Excretion of Nitrates and Nitrites. Urines were collected in metabolic cages. Two samples of 2-h intervals were obtained; the first, from hours 2 to 4 after CYP and the second, from hours 4 to 6 after CYP. Urinary volumes were measured, and the urine samples were frozen at −60°C until assayed. After protein precipitation, nitrates were quantitatively converted to nitrites by the action of the nitrate reductase (obtained from *Klebsiella pneumoniae*) for 60 min under anaerobic conditions. The total nitrite concentration was then estimated by the Griess reaction and read at 540 nm in a spectrophotometer. Urine samples were processed in duplicates. Creatinine was quantified by a modification of the Jaffe reaction, with picric acid in alkaline solution. NO metabolites (nitrates + nitrites) were expressed as millimoles per gram of creatinine.

NOS Activity. NOS activity was measured in the rat bladder as the formation of L-[^{14}C]citrulline from L-[^{14}C]arginine (NEN Life Science Products, Wilmington, DE) (Salter et al., 1991). The bladders were minced and suspended in 10 volumes of cold 20 mM HEPES buffer (pH 7.4), containing 1 mM dithiothreitol and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml aprotinin). Bladders were homogenized by a Polyton (Brinkmann Instruments, Westbury, NY) and subsequently centrifuged at 20,000*g* for 20 min, at 4°C. Endogenous L-arginine was removed by passing the supernatants through a 0.5-ml column of AG 50W-X8, Na^+ form (Bio-Rad, Hercules, CA). Supernatants were incubated in the presence of 3 μM L-[^{14}C]arginine (3 μM final concentration), 10 mM valine, 10 μM tetrahydrobiopterin, 10 μM FAD, 2 mM NADPH, in the presence of 1 mM EGTA. The reaction was terminated by the addition of 1 ml of 20 mM HEPES buffer (pH 5.5) containing 2 mM EDTA, and the sample was immediately passed through an AG 50W-X8, Na^+ form column (1 ml) and eluted with HEPES (pH 5.5). The radioactivity was determined by liquid scintillation spectrometry. iNOS is the calcium-calmodulin-independent isoform of NOS (Moncada et al., 1991). Therefore, iNOS activity was calculated from the differences between samples containing EGTA (3 mM) and samples containing the NOS inhibitor, L-NMMA (1 mM). Protein concentrations were measured as described by Bradford (1976). NOS activity was expressed as picomoles of citrulline per milligram of protein per minute.

CYP was purchased from Sigma Chemical Co. (St. Louis, MO), and L-NNA was from Research Biochemicals International (Natick, MA). GR215070 was kindly donated by Glaxo Wellcome Laboratories (Greenford, UK).

Statistical Analysis. All the results are expressed as mean ± S.E., and statistical significance was determined by ANOVA followed by a post hoc Duncan's test. Two-group analysis was determined by Student's *t* test. Differences were considered significant at $P < .05$.

Results

The contents of Evans blue, expressed as micrograms per gram of tissue, in control rats and the effects of CYP are shown on Fig. 1. CYP induced a marked increase (30- to 40-fold increase above control levels) in protein plasma extravasation in the rat urinary bladder. Protein plasma extravasation was significantly greater at 6 than at 4 h after CYP (Fig. 1). The effects of CYP on iNOS activity and on NO production are shown in Tables 1 and 2 and Fig. 1. Calcium-independent NOS activity, characteristic of iNOS, was undetectable in bladders from control rats; however, 6 h after treatment with CYP there was a marked increase in calcium-independent NOS activity (Table 2). Urinary NO metabolites (nitrates + nitrites) were quantitated in urines collected from 2 to 4 h and from 4 to 6 h after CYP. Administration of CYP increased the urinary excretion of NO metabolites with greater increases in samples collected from 4 to 6 h, than from 2 to 4 h (Fig. 1). Higher excretion of NO metabolites was

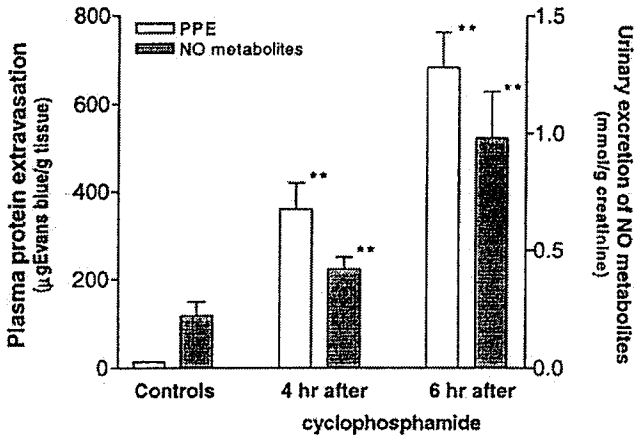

Fig. 1. Effects of CYP on urinary bladder plasma protein extravasation and on the urinary excretion of NO metabolites. Rats were sacrificed either at 4 or at 6 h after i.p. administration of 150 mg/kg CYP. Rats were placed on metabolic cages for urine collection, and urines were collected from 2 to 4 h and from 4 to 6 h after CYP. Subsequently, the rats were anesthetized by the i.p. administration of urethane (1.2 g/kg). Evans blue dye (50 mg/kg) was injected via the jugular vein, and 15 min later the rat was exsanguinated. The urinary bladder was removed and blotted dry before the Evans blue dye accumulation on the bladder was determined and expressed as micrograms of Evans blue per gram of tissue. The NO metabolites (nitrates + nitrites) were quantitated on the urine samples (2–4 h and 4–6 h). Left ordinate: plasma protein extravasation as micrograms of Evans blue/g of tissue. Right ordinate: urinary excretion of NO metabolites as millimoles of nitrates + nitrites/g of creatinine. Shown are mean values ± S.E. of at least six rats per group. **, significantly different from control values at $P < .01$.

TABLE 1

Effects of CYP on the urinary excretion of NO metabolites: interaction with a NO synthesis inhibitor and a selective NK_1 receptor antagonist

CYP was given as a single dose of 150 mg/kg, i.p. L-NNA (10 mg/kg, i.p.) was administered as a single dose, 5 min before CYP. GR-205171 (10 mg/kg, i.p.) was given either as a single dose 5 min before CYP or as two doses, one 5 min before and the second 3 h after the cytotoxic. No metabolites (nitrates + nitrites) were quantitated in urine collected between the 2nd and 4th h after administration of CYP.

	Urinary Excretion of NO Metabolites
	mmol/g creatinine
Control	0.22 ± 0.08
GR-205171	0.18 ± 0.08
L-NNA	0.05 ± 0.04**
CYP	0.42 ± 0.05**
CYP + GR-205171 (one dose)	0.38 ± 0.1
CYP + GR-205171 (two doses)	0.22 ± 0.08##
CYP + L-NNA	0.11 ± 0.05##
CYP + GR-205171 (two doses) + L-NNA	0.14 ± 0.08##

Significantly different from control values at ** $P < .01$.
Significantly different from CYP values at ## $P < .01$.

associated with greater plasma protein extravasation (Fig. 1).

Neither L-NNA (10 mg/kg, i.p.), an inhibitor of NO synthesis, nor GR205171 (10–20 mg/kg, i.p.), a selective NK_1 antagonist, had any significant effect per se on the basal levels of protein plasma extravasation (not shown). However, GR205171 reduced CYP-induced protein plasma extravasation, with two doses being more effective than a single dose of the NK_1 antagonist (Figs. 2 and 3). Similarly, L-NNA (10 mg/kg, i.p.) exerted a protective effect on CYP-induced protein plasma extravasation. The bladder content of Evans blue after CYP was reduced by 40 to 50% by treatment with L-NNA (Figs. 2 and 3). The effects of combined treatment with GR205171 and L-NNA on protein plasma extravasation

TABLE 2

Effects of CYP on iNOS activity in the rat bladder

Rats were treated either with saline (0.1 ml/100 g, i.p.) or CYP at a dose of 150 mg/kg, i.p. Animals were sacrificed 6 h after administration of saline or CYP. Bladder was removed and assayed for NOS activity. Calcium-independent NOS activity in the supernatant of bladder homogenates was assayed by the rate of conversion of labeled L-arginine to labeled L-citrulline (see *Materials and Methods* for details). The results are expressed as mean values ± S.E. ($n = 5$).

Treatment	iNOS Activity
	pmol citrulline/mg protein/min
Saline	0.5 ± 1
CYP	12.6 ± 2*

* Significantly different from controls (saline treated) rats at $P < .001$.

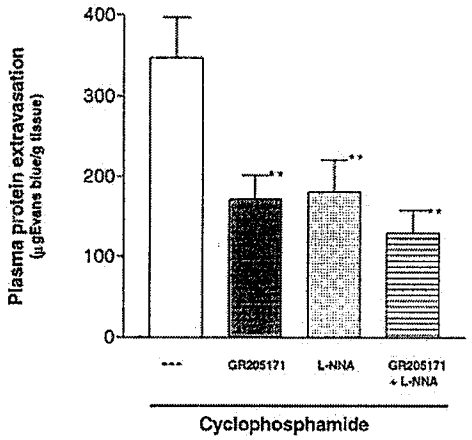

Fig. 2. Effects of GR205171 and L-NNA on CYP-induced plasma protein extravasation. Experiments were conducted as described in the legend for Fig. 1. Results from rats sacrificed only 4 h after CYP administration are depicted in this graph. GR205171 was administered i.p. in two doses of 10 mg/kg each (one 5 min before the CYP and the second, 3 h later). L-NNA (10 mg/kg, i.p.) was given 5 min before the CYP administration. Shown are mean values ± S.E. of at least six rats per group. **, significantly different from control values at $P < .01$. No significant differences were observed between GR205171, L-NNA, and GR205171 + L-NNA groups.

are shown in Figs. 2 and 3. Combined treatment with the NK_1 antagonist and the NO-synthesis inhibitor produced comparable reduction in protein plasma extravasation to those achieved with each drug given separately. No significant differences were observed between these groups. No additive effects were observed with the drug combination.

L-NNA markedly decreased the basal urinary excretion of NO metabolites, as well as CYP-induced increases in urinary NO metabolites (Table 1). The increases in NO metabolites induced by CYP were also reduced by GR205171, with two doses being more effective than a single dose of GR205171 (Fig. 4). GR205171 had no effect on basal NO-metabolite excretion.

The histological appearance of bladders obtained from control animals (group 1), animals treated with CYP (group 4), and of rats treated with CYP + L-NNA + GR205171 (group 8), are shown on Fig. 5. CYP induced marked urothelial damage, edema, vascular congestion, and white blood cell infiltrate (Fig. 5B). Combined treatment with L-NNA and GR205171 reduced the histological damage and the inflammatory changes induced by CYP in the rat bladder (Fig. 5C). Lesser edema, congestion, and white blood cell infiltrates were observed in the group treated with the NO synthesis inhibitor and NK_1 antagonist.

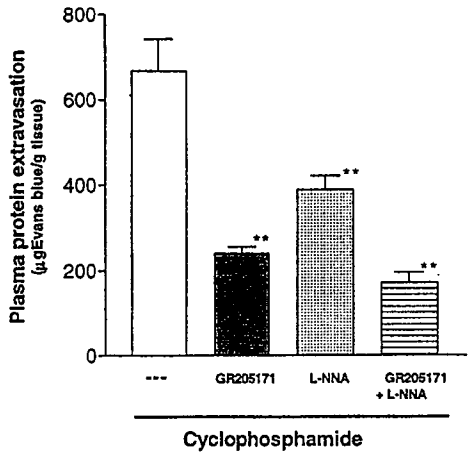

Fig. 3. Effects of GR205171 and L-NNA on CYP-induced plasma protein extravasation. Experiments were conducted as described in legend for Fig. 1. Results from rats sacrificed only 6 h after CYP administration are depicted in this graph. GR205171 was administered i.p. in two doses of 10 mg/kg each (one 5 min before the CYP and the second, 3 h later). L-NNA (10 mg/kg, i.p.) was given 5 min before the CYP. Shown are mean values ± S.E. of at least six rats per group. **, significantly different from control values at $P < .01$. No significant differences were observed between GR205171 and GR205171 + L-NNA groups. Values obtained with GR205171 + L-NNA were significantly lower than those obtained with L-NNA alone ($P < .05$) but not from values obtained with GR205171 alone.

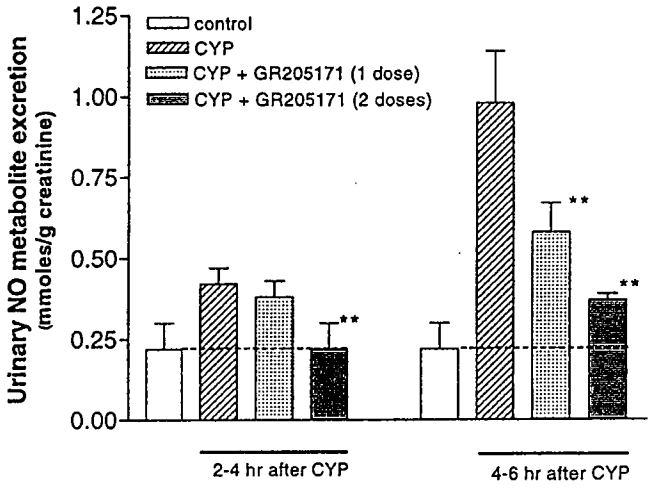

Fig. 4. Effects of CYP on NO metabolite excretion: interaction with GR205171. Experiments were conducted as described in the legend for Fig. 1. Urinary excretion of NO metabolites (nitrates + nitrites), expressed as millimoles of NO metabolites per gram of creatinine, was quantified in urine samples collected from 2 to 4 h and from 4 to 6 h after CYP. Urinary NO metabolites were also measured in control rats, 2 to 4 h and 4 to 6 h after being treated with saline. Dotted lines across the figure were drawn to facilitate observing changes above control values. Shown are mean values ± S.E. of at least six rats per group. **, significantly different from CYP-treated rats at $P < .01$.

Discussion

CYP is known to produce hemorrhagic cystitis (Grimberg-Funes et al., 1990). In the present study, treatment with CYP produced marked plasma protein extravasation, vascular congestion and edema of the bladder, extensive leukocyte infiltration, and damage of the urothelium. Interestingly, these changes were associated with the appearance of calcium-independent NOS activity, which characterizes iNOS expression, and with increases in the urinary excretion of NO metabolites. These findings together with the observation that treatment with NOS inhibitors markedly ameliorates CYP-induced cystitis, indicates a fundamental role for NO in the pathogenesis of this form of drug-induced toxicity.

Current evidence indicates that pretreatment with selective NK_1 receptor antagonists ameliorates CYP-induced cystitis. Pretreatment with RP67580 (Ahluwalia et al., 1994), GR203050 (Alfieri and Gardner, 1997), and GR205171 (present study) ameliorated plasma protein extravasation and the histological damage of the urinary bladder of rats and ferrets treated with CYP. In addition, pretreatment with capsaicin has been shown to reduce CYP-induced cystitis (Ahluwalia et al., 1994). These observations indicate that part of the inflammatory changes induced by CYP in the urinary bladder is mediated via the activation of NK_1 receptors. Consequently, drugs with antagonistic activity on NK_1 receptors are expected to be effective against this toxicity. Although the intrinsic mechanisms accounting for NK_1 activation are not fully established, neuropeptides (sP and neurokinin A) released from PACSF may be involved in this process. This view is supported by the observation that acute administration of capsaicin as well as a variety of chemical stimuli (i.e., xylene) increases plasma protein extravasation in the bladder, only when the PACSF are intact (Maggi and Meli, 1988). In conclusion, our findings support the view that activation of NK_1 receptors, possibly by neuropeptides released from PACSF, plays a role in the pathogenesis of the inflammatory cystitis induced by CYP.

In addition to attenuating plasma protein extravasation and the histological changes indicative of inflammatory cystitis (Alfieri and Cubeddu, 1997; Alfieri and Gardner, 1997), the NK_1-tachykinin receptor antagonist markedly reduced the increase in urinary NO metabolites induced by CYP (present study). Our results suggest that the increase in NO metabolites occurring within the first 6 h of CYP administration derives mainly from activation of NK_1 receptors, possibly by sP and/or related neuropeptides. It thus appears that NK_1-mediated NO formation plays a pathogenic role in this form of toxicity. Frode-Saleh and colleagues (1999) have also shown that NO mediates sP-induced inflammatory changes in animal models of pleurisy. Interestingly, in our model GR205171 did not affect the basal levels of NO metabolites, and it was only effective in reducing the increases in NO metabolites induced by CYP. These results indicate that GR205171 does not exert a nonspecific effect on NOS activity.

Although we can not determine what proportion of the urinary NO metabolites derives from the inflamed bladder, the fact that bladders from CYP-treated rats showed a marked increase in calcium-independent conversion of L-arginine to L-citrulline, characteristic of an increased iNOS activity, suggests that at least part of the increase in urinary excretion of NO metabolites represents increased bladder production of NO. Increased urinary levels of NO metabolites have also been observed in other animal models of chemical cystitis, despite having negative cultures (Lundberg, 1996). In conclusion, iNOS activity is increased in bladders of CYP-

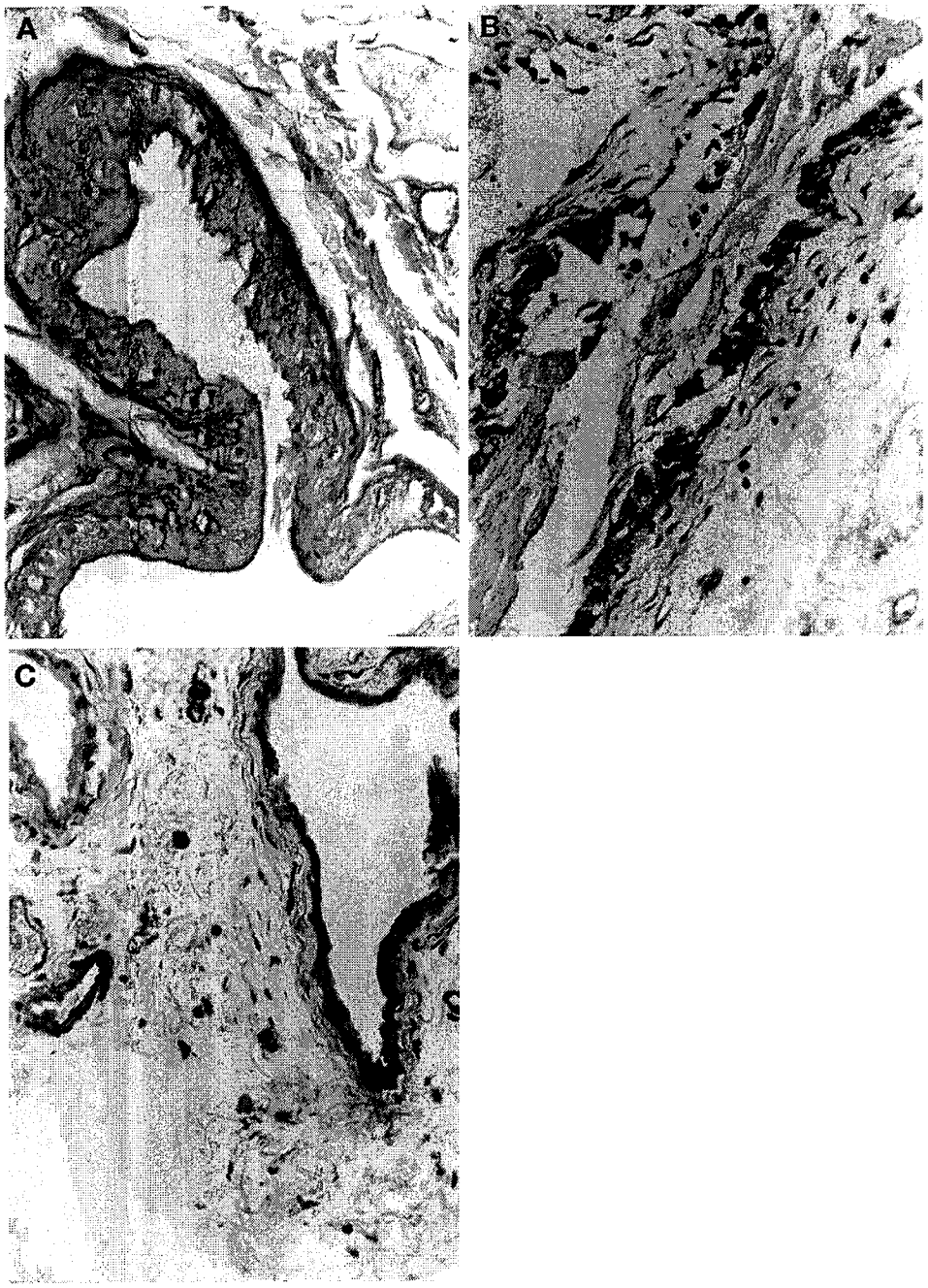

Fig. 5. Histological changes in the rat bladder induced by CYP: effects of combined treatment with GR205171 and L-NNA. All sections were stained with H&E and photographed at 25×. A, control rats (group 1, saline injection): intact urothelium, blood vessels with thin walls, no edema or infiltrate present (0/+++), two smooth muscle layer without alterations. B, CYP rats (group 4): extensive urothelial damage, urothelium was absent from many regions of the bladder, marked inflammatory infiltrate with abundant lymphocytes, and polymorphonuclear white blood cells (+++/++++), marked edema separating the smooth muscle layers, and marked congestion. C, CYP + L-NNA + GR205171 (group 8): urothelium present, mild to moderate vascular congestion, mild edema, and few white blood cell infiltrates (+/++++). C shows considerable improvement in comparison with histological findings of B.

treated rats, where it plays an important role in increasing the local production of NO, which is an important mediator of the inflammatory bladder damage.

In conclusion, the following mechanism is proposed to explain the inflammatory cystitis produced by CYP. Acrolein, its major metabolite, seems responsible for a large part of the bladder toxicity observed during CYP treatment (Phillips et al., 1961; Cox, 1979; Fraiser et al., 1991). The parent drug and/or its metabolites (acrolein) are concentrated in the urine, reaching the bladder where they would stimulate the PACSF, leading via antidromic stimulation to the release of neuropeptides, such as sP and neurokinin A, which would in turn activate NK_1 receptors (NK_2?), enhancing NO production and release. Increased vasodilation and vascular permeability, together with a possible direct irritant stimulus (acrolein), should lead to white blood cell and mast cell infiltration, leading to a local increase in the production of cytokines. The cytokines would further stimulate NO production through further induction of iNOS. High levels of NO may also sensitize the PACSF to further enhance neuropeptide release, leading to a positive feedback loop of inflammation. Blockade of NK_1 receptors or inhibition of NO synthesis would reduce NO production, and thus decrease inflammatory damage to the bladder. However, it is clear that additional undefined mechanisms are also involved in CYP-induced cystitis, because neither the NK_1 antagonist nor the

NO synthesis inhibitor, either alone or in combination, were able to completely prevent the toxicity.

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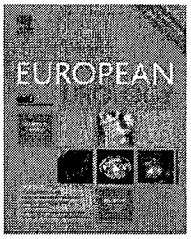

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Editorial

Tachykinins: Role in Detrusor Overactivity?

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The nerves of the lower urinary tract synthetize, store, and release many neuropeptides, including tachykinins, i.e., substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) [1]. It is generally accepted that tachykinins have an important role in afferent signaling from the bladder, and that they may be involved in different pathologies in the lower urinary tract. A pathophysiologic role in detrusor overactivity (DO) and the overactive bladder (OAB) syndrome has been suggested [2], but has not been established. Recently, Sellers et al. [3] found NKA-induced responses to be impaired in detrusor muscle from patients with idiopathic DO, but not from patients with neurogenic DO, in whom the response did not differ from that observed in detrusor tissue from normal controls. Even if the observation of Sellers et al. [3] supports their conclusion that idiopathic and neurogenic DO may have different pathophysiology (which does not seem unreasonable), it does not clarify the potential role of tachykinins in the pathogenesis of DO/OAB. If these peptides have such a role, where is the site of action: the detrusor muscle, afferent nerves, or other structures within the bladder? Or is their main site of action not in the bladder, but in the central nervous system (CNS)?

1. Tachykinins and tachykinin receptors

Tachykinins act on specific, G-protein–coupled neurokinin (NK) receptors, and SP, NKA, and NKB possess the highest affinity for NK1, NK2, and NK3 receptors, respectively. All receptor subtypes have been identified in urinary bladders of various mammals, both in vitro and in vivo [4]. In the rat detrusor, NK1, NK2, and NK3 receptors have been demonstrated, as evidenced by radioligand binding, autoradiographs, and functional experiments, whereas, in hamster, mouse, dog, and human detrusor, NK2 receptors predominate [4]. The nerves containing the tachykinins are localized mainly suburothelially, but also can be found within the detrusor muscle. Binding sites for the peptides are localized mainly to the detrusor muscle, but also can be demonstrated either directly or functionally on blood vessels and on the urothelium/suburothelium [1].

2. Afferent signaling and "efferent" functions

Tachykinins are believed to serve not only as mediators of afferent functions, but they may also have a local effector or efferent function [4]. Thus, they may act as neurotransmitters and/or neuromodulators in the bladder ganglia and at the neuromuscular junctions. As a result, these peptides can be involved in the mediation of various effects, including smooth muscle contraction, potentiation of efferent neurotransmission, changes in vascular tone and permeability ("neurogenic inflammation"), and micturition reflex activation [4]. Evidence for their role is based mainly on experiments in animals.

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3. Detrusor contraction

In the human detrusor, the presence of tachykinins, their receptors, and their contractile effects have been well documented [5]. The predominant tachykinin receptor mediating contraction of the human detrusor is of the NK2 type.

The NK2 receptor–mediated contraction in the detrusor is dependent largely on the activation of L-type Ca^{2+} channels and is sensitive to nifedipine [6]. However, a role of intracellular Ca^{2+} cannot be excluded, since NK2-receptor stimulation also activates phospholipase C [6]. Prostanoids generated after NK2 receptor activation may amplify the direct contractile effect of NK2-receptor stimulation [4]. Additional mechanisms may be involved, Thus, possible attenuation of NKA-induced contractions of the rat detrusor by the Rho-kinase inhibitor Y-27632 suggests involvement of the Rho-kinase pathway [5,6].

Contractions of the detrusor may involve both cholinergic and noncholinergic components [5]. Even if the noncholinergic component seems to be mediated mainly by ATP, involvement of other mediators has not been completely excluded [5]. Whether tachykinins can contribute to the atropine-resistant contraction has been unclear. However, no involvement of tachykinins has been demonstrated either in contraction of normal human detrusor tissue, where the atropine-resistant component of responses to electrical stimulation of nerves is small, or in detrusor tissue from patients with e.g., idiopathic DO [7], where the atropine-resistant component can amount to 50% [5]. This finding, together with the observation of Sellers et al. [3] that the sensitivity to NKA is decreased in idiopathic DO, does not favour the view that a direct contractile effect of tachykinins on the detrusor muscle is involved in the generation of DO/OAB.

4. Central control

SP, NKA, NKB, and their preferred receptors, NK1, NK2, and NK3, respectively, have been demonstrated in various CNS regions, including those involved in micturition control [4]. NK1 receptor–expressing neurons in the dorsal horn of the spinal cord may play an important role in DO/OAB. Thus, at the spinal level, there was a tachykinin involvement via NK1 receptors in the micturition reflex induced by bladder filling. This was demonstrated in normal rats, and more clearly, in rats with bladder hypertrophy secondary to bladder outflow obstruction [5].

Seki et al. [8] demonstrated that NK1 receptor-expressing neurons in the spinal cord could be eliminated by using intrathecal substance P-saponin conjugate (SSP-SAP). They found that SSP-SAP reduced capsaicin–induced DO and suggested that SSP-SAP could be effective in treating DO induced by bladder irritation without affecting normal bladder function. In conscious rats undergoing continuous cystometry, antagonists of both NK1 and NK2 receptors inhibited micturition, decreasing micturition pressure and increasing bladder capacity at low doses, and inducing dribbling incontinence at high doses. This effect was most conspicuous in animals with outflow obstruction [5].

5. Tachykinins and detrusor overactivity

A significant increase in the density of suburothelial, SP-containing nerves was found in patients with idiopathic DO, compared with stable controls [9,10]. Since capsaicin-sensitive afferents (containing tachykinins) may be a part of a spinal, vesicovesical excitatory (short-loop) reflex providing a neurogenic mechanism for overactive detrusor contractions, both idiopathic and neurogenic [5], it cannot be excluded that peripheral tachykinins may be involved in pathophysiologic afferent signaling associated with DO/OAB. However, despite promising effects in animal models [5], there seem to be no published proof of concept studies showing that any of the selective NK-receptor antagonists available has a therapeutic effect in patients with DO/OAB.

6. Conclusions

Even if it has been suggested that the lower urinary tract is a primary site of action of the tachykininergic system, the roles of tachykinins in different types of bladder dysfunction still remain to be established. The investigation of Sellers et al. [3] shows that their effects on the detrusor muscle in different types of DO may not be the same. We do not know whether the authors' findings reflect whether the reaction to the peptides is changed as a consequence of other factors related to the disorder, or whether the bladder tachykinins are involved directly in the generation of some types of DO. A role of tachykinins within the CNS in the pathogenesis of DO cannot be excluded and should be further explored.

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Efficacy and Safety of a Neurokinin-1 Receptor Antagonist in Postmenopausal Women With Overactive Bladder With Urge Urinary Incontinence

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Purpose: Urge urinary incontinence is the involuntary leakage of urine commonly occurring in older adults, particularly women. Preclinical evidence suggests that urge urinary incontinence may occur due to up-regulation of tachykinin mediated bladder/spinal reflex signaling. This study tested the hypothesis that aprepitant, a neurokinin-1 receptor antagonist, may be efficacious in the treatment of urge urinary incontinence.

Materials and Methods: This was a double-blind, randomized, placebo controlled, parallel group pilot study in which postmenopausal women with a history of urge urinary incontinence or mixed incontinence (with predominantly urge urinary incontinence) were assigned to receive a 160 mg capsule of aprepitant (61) or placebo (64) once daily for 8 weeks. The primary end point was percent change from baseline in average daily micturitions assessed by a voiding diary. Secondary end points included average daily total urinary incontinence and urge urinary incontinence episodes, and urgency episodes.

Results: Aprepitant significantly decreased the average daily number of micturitions compared with placebo at 8 weeks. The between-group treatment difference expressed as percent change from baseline was −6.8%, 95% CI (−12.5, −1.1) ($p = 0.019$). The average daily number of urgency episodes was also significantly reduced compared to placebo ($p = 0.049$). The average daily number of urge urinary incontinence and total urinary incontinence episodes were also reduced, although the difference was not statistically significant. Aprepitant was generally well tolerated and adverse experiences were generally mild.

Conclusions: Results of this initial study suggest that neurokinin-1 receptor antagonism may represent a novel therapeutic approach to treating overactive bladder syndrome.

Key Words: urinary incontinence; controlled clinical trial; receptors, neurokinin-1; antagonists and inhibitors

Urge urinary incontinence has been described as the involuntary loss of urine accompanied or immediately preceded by urgency (a compelling desire to void that is difficult to defer), consequent to involuntary contraction of the detrusor muscle.[1] UUI has been related to overactive bladder syndrome, a clinical disorder characterized by urgency and/or UUI (so-called OAB dry and OAB wet, respectively), usually with frequency, in the absence of local pathological or endocrine factors. Although differences of opinion exist with regard to terminology, for the purposes of this report we will use UUI and OAB to refer to the common clinical disorder previously described. The prevalence of UUI increases with age, and approximately 19% of women 65 years old or older report at least weekly urgency, frequency and UUI episodes.[2] People with UUI report a decrease in quality of life, including impacts on daily activities, social interactions and perception of health status.[3]

Antimuscarinic drugs, such as oxybutynin and tolterodine, are the most widely prescribed therapy for treatment of OAB syndrome.[4] These drugs act in part by blocking muscarinic receptors in the detrusor muscle decreasing contractility. However, antimuscarinics are limited by poor tolerability due to side effects, such as dry mouth, blurred vision, and constipation. Because the condition may require lifelong therapy, the inability to tolerate antimuscarinic therapy is problematic. Thus, there is a need to develop alternative therapies, not only with better efficacy but also with improved tolerability.

Studies using C fiber neurotoxins suggest that tachykinins, such as substance P, may play a role as sensory transmitters in the micturition reflex. In addition, intravesical capsaicin and resiniferatoxin, believed to work by depleting tachykinins in the bladder, have been reported to demonstrate clinical efficacy. This suggests that NK-1 receptor antagonists may be efficacious in this disorder. Further support for this hypothesis comes from studies using selective NK-1 antagonists in preclinical models.[5]

Aprepitant is a potent, selective, CNS penetrating NK-1 receptor antagonist currently used to treat chemotherapy induced nausea and vomiting. The purpose of this study was to determine whether treatment with aprepitant improves bladder symptoms in postmenopausal women with UUI.

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Study received ethical review committee approval for each study center.

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METHODS

Study Design

This was a double-blind, randomized, placebo controlled, pilot study conducted at 26 sites in the United States between January and August 2003 using in-house blinding procedures. Following screening, and a 1-week single-blind placebo controlled run-in period, patients were randomized to receive a 160 mg tablet of aprepitant or a placebo tablet once daily for 8 weeks during the double-blind period according to a computer generated allocation schedule. The 160 mg daily dose was selected based on positron emission tomography studies demonstrating greater than 95% CNS (striatum) receptor occupancy at this dose.[6] Patients visited the clinic 1, 2, 4 and 8 weeks following randomization. The study was approved by the ethical review committees for each study center and all patients gave written informed consent.

Patients

Patients were females 40 years old or older in good general health with no history of menses for at least 1 year before the study start. Patients had to demonstrate, on average, 8 or more micturitions daily and 1 or more UUI episodes daily in a patient voiding diary during the screening and placebo run-in periods. Patients with mixed (urge and stress) incontinence were eligible if the above criteria were met, and urge incontinence episodes exceeded stress incontinence episodes. Patients refrained from using anticholinergic medications and smooth muscle relaxants for 2 weeks before screening and throughout the study. Patients receiving tricyclic antidepressants were eligible, provided doses were stable during the study and for at least 2 weeks before screening. Patients could be on stable doses of calcium channel blockers or α-adrenergic agonists but could not have taken any drug with a narrow therapeutic window, such as warfarin or digoxin, within 4 weeks of the first visit or be treated with unstable doses of diuretics within 2 weeks before screening. Patients with a history of continual urine leakage or who were unaware of leakage, those who showed no improvement in UUI symptoms with prior anticholinergic therapy, those who were treated within 4 weeks of the first visit with inhibitors or inducers of cytochrome P450 3A4, warfarin or digoxin, or those who had chronic treatment with antihistamines were excluded from study.

Efficacy

During the week before each visit patients recorded micturitions, urgency episodes and incontinence episodes by type (urge, stress or other) in a validated voiding diary.[7] The primary end point was percentage change from baseline (placebo run-in period) in average daily micturitions at week 8. Secondary end points included percentage change from baseline at week 8 in average daily urgency episodes, total urinary incontinence and UUI episodes. Urgency was defined as a strong urge to urinate immediately. UUI episodes were defined as accidental urine leakage because of an urge or pressure to urinate and the patient felt that they could not make it to the bathroom. Patients also completed questionnaires assessing their perception of leakage/urine loss, disease specific quality of life, and urinary symptoms, including the Incontinence Impact Questionnaire (short form),[3] UUDI[8] and the Incontinence Quality of Life Questionnaire.[9]

Safety

Safety analysis included all randomized patients who received at least 1 dose of therapy. Safety and tolerability were assessed by statistical and clinical review of adverse experiences. Clinical evaluations for safety assessment included monitoring AEs, measuring vital signs, physical examinations and ECGs. Laboratory safety tests included blood chemistry, hematology and urinalysis.

Statistical Analysis

The primary efficacy analysis used a modified intent to treat analysis that included all randomized patients who took at least 1 dose of study drug and who had at least 1 posttreatment assessment. Missing data were addressed using a last observation carried forward approach. In the primary analysis treatment comparisons of the percentage change from the baseline in the average number of daily micturitions at week 8 were made using an ANOVA model including terms for treatment and study center. The absolute change from baseline also was described, as supportive analysis. Treatment $\times$ center interaction was investigated at the 10% significance level using an ANOVA and if present, a Gail and Simon test at the 5% significance level was used to evaluate whether the interaction was qualitative.[10] A similar ANOVA model with treatment and study center as factors was used for the secondary hypotheses concerning average daily UUI episodes, total UI episodes, and urgency episodes. Mean treatment differences and 95% CI for primary and secondary end points were calculated. Summary statistics (mean and SD) were provided for each time point. Mean treatment differences and 95% CI for the questionnaires were estimated using an ANOVA model with treatment and study center as factors.

Fisher's exact test was used to compare AEs between treatment groups, and risk differences with corresponding 95% CI were calculated using the method of Miettinen and Nurminen.[11] An exploratory analysis using ANOVA was performed for the primary and secondary end points of percent change and change from baseline at week 8 based on whether patients were naïve or non-naïve to any type of anticholinergic therapies. Since this was a pilot proof of concept study no adjustments for multiplicity were planned.

A sample size of 47 evaluable patients per group provided approximately 80% power to detect a 10.0 percentage point difference between groups with respect to the percent

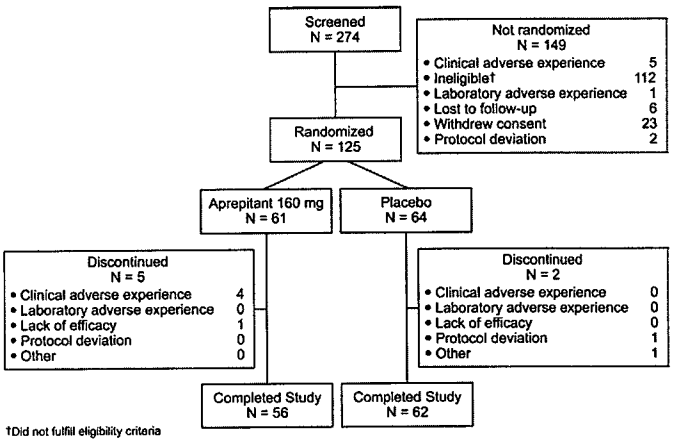

FIG. 1. Patient disposition

TABLE 1. *Patient demographics and baseline characteristics*

	Aprepitant	Placebo
Mean pt age ± SD	65.1 ± 11.0	64.5 ± 8.8
No. race (%):		
White	55 (90.2)	59 (92.2)
Black	4 (6.6)	2 (3.1)
Asian	1 (1.6)	0 (0.0)
Hispanic-American	1 (1.6)	3 (4.7)
Mean yrs UUI ± SD	8.6 ± 9.2	8.2 ± 8.1
Mean daily micturitions ± SD*	11.1 ± 2.5	10.4 ± 2.2
Mean total incontinence episodes ± SD*	3.5 ± 2.5	3.3 ± 2.3
Mean urge incontinence episodes ± SD*	3.2 ± 2.3	3.2 ± 2.2
Mean urgency episodes ± SD*	8.2 ± 3.4	7.3 ± 3.7
No. pts receiving prior anticholinergic therapy (%)	40 (65.6)	42 (65.6)

* Baseline derived from placebo run-in diary. Average daily number calculated as sum of occurrences divided by number of days of diary completion.

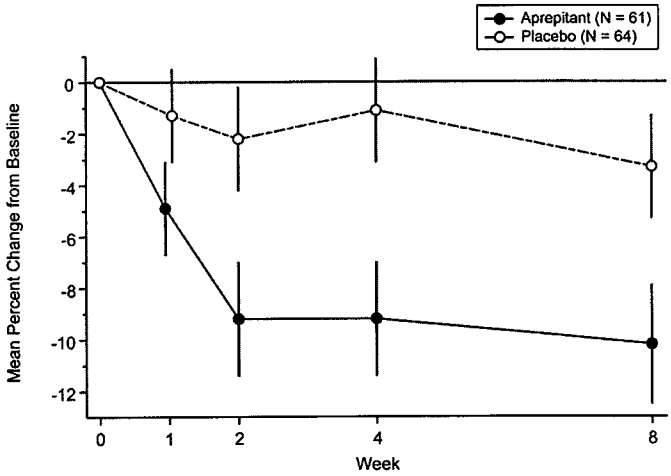

FIG. 2. Percent change from baseline in average daily micturitions (±1 SE) during 8 weeks of treatment for aprepitant and placebo groups. Average number of daily micturitions is calculated as sum of occurrences during diary week (4 to 10 days) divided by number of days of diary completion.

change from baseline in the number of daily micturitions averaged during a diary card week at week 8. To account for attrition approximately 65 patients per arm were planned for enrollment.

RESULTS

Patients

Of the 274 patients screened 125 were randomized and 118 completed the study (fig. 1). The mean age was 64.8 ± 9.9 years (range 40 to 91) and the mean duration of UI was 8.4 ± 8.7 years (table 1). Approximately 65% of patients in both groups had received prior anticholinergic therapy. There were no significant differences between the groups for any baseline characteristic.

Efficacy

There was a significant reduction in average daily micturitions change from baseline for aprepitant (−10.2% ± 17.9%) compared with placebo (−3.3% ± 16.3%), and the estimated mean difference was significant (p = 0.019) (table 2). Expressed as the absolute number of events, the change from baseline for aprepitant was −1.3 ± 1.9 compared with −0.4 ± 1.7 for placebo and the estimated difference was significant (p = 0.003) (table 2). The benefit of aprepitant in reducing daily micturitions was consistent during the 8-week study period (fig. 2).

There was a significant percentage reduction in average daily urgency episodes in the aprepitant group compared with that for placebo (estimated difference −13.2%; 95% CI: −26.3, 0.1; p = 0.049) as well as in the absolute change from baseline (estimated difference −1.2; 95% CI: −2.1, −0.3; p = 0.007) (table 2). The reduction in daily urgency episodes was consistent during the 8 weeks of therapy (fig. 3). Reductions in favor of aprepitant were also seen for the average number of daily UUI episodes and total UI episodes, although these differences did not reach statistical significance. A subgroup analysis of patients who had received prior anticholinergic therapy demonstrated results similar to those observed for the total population (data not shown).

Aprepitant significantly improved patient perception of leakage (p = 0.002) and the degree of bother associated with urinary symptoms (UUDI, p = 0.035) compared with results for placebo (table 3). Patient disease specific quality of life scores were not statistically different between the groups (table 3).

Safety

Overall 36 (59%) patients in the aprepitant group and 18 (28.1%) patients in the placebo group had at least 1 clinical

TABLE 2. *Change in efficacy end points (sum of occurrences during diary week divided by number of days of diary completion) at 8 weeks of treatment*

Daily Occurrence of End Points	Mean ± SD		Estimated Treatment Difference* Between Aprepitant + Placebo (95% CI)	p Value
	Aprepitant	Placebo		
Micturitions:				
% Change from baseline	−10.2 ± 17.9	−3.3 ± 16.3	−6.8 (−12.5, −1.1)	0.019
Change from baseline	−1.3 ± 1.9	−0.4 ± 1.7	−0.9 (−1.5, −0.3)	0.003
Urge incontinence episodes:				
% Change from baseline	−50.4 ± 45.2	−35.6 ± 44.4	−14.5 (−30.2, 1.1)	0.070
Change from baseline	−1.5 ± 1.6	−1.1 ± 2.0	−0.4 (−1.0, 0.3)	0.234
Total incontinence episodes:				
% Change from baseline	−48.3 ± 45.9	−36.3 ± 43.7	−14.8 (−27.6, 4.1)	0.145
Change from baseline	−1.5 ± 1.7	−1.2 ± 2.1	−0.3 (−1.0, 0.3)	0.323
Urgency episodes:				
% Change from baseline	−23.2 ± 32.2	−9.3 ± 40.0	−13.2 (−26.3, −0.1)	0.049
Change from baseline	−1.8 ± 2.5	−0.5 ± 2.6	−1.2 (−2.1, −0.3)	0.007

* Computed from a statistical model adjusting for investigator sites.

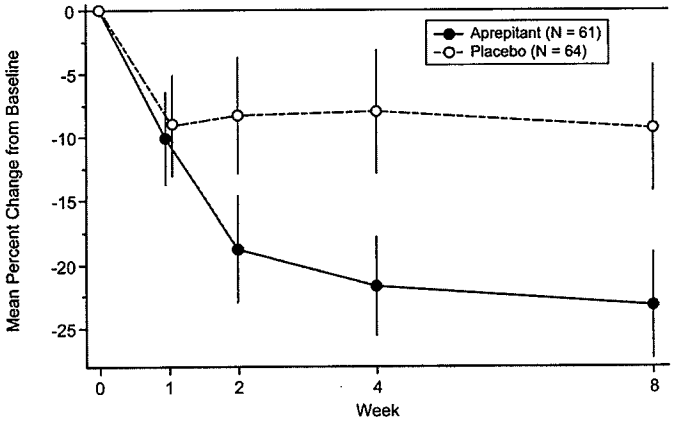

FIG. 3. Percent change from baseline in average daily number of urgency episodes (±1 SE) during 8 weeks of therapy for aprepitant and placebo groups. Average number of daily urgency episodes is calculated as sum of occurrences during diary week (4 to 10 days) divided by number of days of diary completion.

AE. In the aprepitant group 4 patients had a serious clinical AE (pneumonia [2], palpitations [1] and exacerbation of chronic obstructive lung disease [1]), 3 of which resulted in discontinuation and an additional patient discontinued therapy due to asthma. One serious AE (basal cell carcinoma) occurred in a patient in the placebo group. The risk difference for discontinuation for aprepitant (4 patients) compared with that for placebo (0 patients) was significant ($p = 0.037$). Only the incidence of palpitations in the aprepitant group (a 60-year-old woman who was hospitalized and evaluated) was considered drug related. A cardiac stress radionuclide study was reported as positive, although serial electrocardiogram, cardiac enzymes and a subsequent cardiac catheterization were within normal limits. Dry mouth, fatigue, pneumonia, urinary tract infection and somnolence were the most commonly reported AEs (table 4). Four cases each of dry mouth, fatigue and somnolence in the aprepitant group and 1 case of dry mouth in the placebo group were considered related to therapy. Clinical AEs considered drug related occurred more frequently in the aprepitant group (18 [29.5%]) than in the placebo group (3 [4.7%]) ($p = 0.001$).

TABLE 3. *Change from baseline treatment differences in end points*

	Estimated Treatment Difference Between Aprepitant + Placebo (95% CI)	p Value
Urinary Incontinence Global Questionnaire:*		
Leakage or urine loss	0.5 (0.2, 0.9)	0.002
Nocturnal symptoms	0.0 (−0.3, 0.4)	0.905
Control of bladder	0.3 (−0.0, 0.6)	0.066
Incontinence Quality of Life Questionnaire:*		
Avoidance + limiting behavior	0.1 (−0.2, 0.4)	0.465
Psychosocial impacts	0.0 (−0.2, 0.2)	0.902
Social embarrassment	0.2 (−0.2, 0.5)	0.311
Incontinence Impact Questionnaire Short Form:†		
Adverse impact	−0.1 (−0.3, 0.2)	0.515
UUDI:†		
Degree of bother	−0.3 (−0.6, 0.0)	0.035

* Positive response indicates improvement.
† Negative response indicates improvement.

TABLE 4. *Clinical adverse experiences occurring in 5% or more of patients*

	Aprepitant	Placebo
Dry mouth*	4 (6.6)	1 (1.6)
Fatigue*	4 (6.6)	1 (1.6)
Pneumonia	4 (6.6)	0 (0.0)
Urinary tract infection	4 (6.6)	1 (1.6)
Somnolence*	4 (6.6)	0 (0.0)

* Considered related to therapy.

Two (3.3%) patients in the aprepitant group and 3 (4.7%) in the placebo group had a laboratory AE. In the aprepitant group 1 patient had increased alanine aminotransferase and aspartate aminotransferase levels, and 1 patient had decreased neutrophil and platelet counts. In the placebo group 2 patients had increased alanine aminotransferase levels and 1 had increased blood glucose levels. None of the laboratory AEs was serious or caused discontinuation from the study. There were no notable differences in vital signs, electrocardiogram or other physical observations between the aprepitant and placebo groups.

DISCUSSION

To our knowledge this is the first clinical trial to demonstrate efficacy for an NK-1 receptor antagonist in UUI. The overall magnitude of treatment benefit is similar to those reported for anticholinergics, the current standard of therapy in OAB/UUI. Anticholinergics have shown a placebo subtracted reduction in the range of −0.5 to −1.3 average daily micturitions after treatment for 12 weeks, with baseline values ranging from 10.1 to 12.9 average daily micturitions.[12,13] Aprepitant also significantly reduced the incidence of urgency episodes during the 8 weeks of treatment. In addition, aprepitant was superior to placebo on patient perceived improvement as well as bother of urinary symptoms. The consistency of the data supports the efficacy of an NK-1 receptor antagonist in treatment of UUI.

Micturition is controlled by neural circuits in the brain and spinal cord.[14] The reasons for urgency are not fully understood and may involve mechanisms of the CNS, afferent neural factors, or detrusor muscle functioning, including micromotions and myofibroblast activity.[15] The nature of the micturition reflex circuitry involves a multitude of neural pathways and transmitters that may become more or less important in pathological situations. In spinal injury of animals and humans, the normally undetectable C fiber micturition reflex appears to become prominent. Studies in animals using C fiber neurotoxins, such as capsaicin and resiniferatoxin, have suggested that tachykinins have a role in the micturition reflex.[16] Substance P is a mammalian tachykinins that may function as a sensory neurotransmitter and is the preferred agonist for NK-1 receptor. NK-1 and neurokinin-2 receptor antagonists may act like capsaicin by inhibiting sensorial input from the bladder to the spinal cord, thus increasing the threshold to initiate micturition.[17] Studies in animals indicate NK-1 receptor antagonists may increase bladder storage without inhibiting voiding.[5] Interestingly NK-1 antagonists may work centrally at the level of the spinal cord, differentiating this mechanism from that of antimuscarinics or spasmolytics.[18,19] Results from studies using NK-1 and neurokinin-2 antagonists in animal models

provided the rationale for the use of the NK-1 receptor antagonist aprepitant to treat UUI.

The incidence of clinical AEs was higher in the aprepitant group than in the placebo group, although in general, AEs were mild and unlikely to be of clinical significance. Episodes of dry mouth, fatigue and somnolence were considered related to aprepitant therapy, however, no AEs resulted in interruption of treatment. Mechanism based side effects, such as dry mouth, which affected 23% to 60% of patients participating in clinical trials of anticholinergic medications, is reported to be the major reason for discontinuation of these therapies.[20] In the current study 4 (6.6%) patients experienced dry mouth, considerably lower than that reported for antimuscarinics. Other AEs were generally mild and did not raise significant safety concerns, consistent with the overall safety and tolerability reported in clinical trials.[21]

Results of this study cannot be extrapolated to other populations, including those with less severe UUI, men, or premenopausal women. Future studies comparing these classes of drugs would be needed to estimate relative incidence and to define the safety and tolerability profiles of aprepitant in the OAB/UUI population.

Voiding diaries are frequently the primary tool used to evaluate efficacy in clinical trials of UUI.[22] The voiding diary used in this study has shown to be a reliable and valid method for assessing change in symptoms of overactive bladder.[7] Although self-monitoring in diary reporting may cause patients to have an enhanced awareness of symptoms during initial completion of the voiding diary (resulting in an apparent early decrease in symptoms), diary eligibility criteria regarding micturitions and UUI episodes were assessed before, and at the end of, the run-in period to minimize the possibility of self-monitoring and large placebo effects often seen in UUI studies.

CONCLUSIONS

Aprepitant, an NK-1 receptor antagonist, significantly improved symptoms of UUI in postmenopausal women. Aprepitant was generally well tolerated and the incidence of side effects, including dry mouth, was low. Results of this initial study suggest that NK-1 receptor antagonism holds promise as a potential treatment approach for OAB. Additional studies are warranted to verify the usefulness of this class of drugs as a novel treatment for OAB.

Abbreviations and Acronyms

AE	=	adverse experience
CNS	=	central nervous system
NK-1	=	neurokinin-1
OAB	=	overactive bladder
UI	=	urinary incontinence
UUDI	=	Urge Urogenital Distress Inventory
UUI	=	urge urinary incontinence

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EDITORIAL COMMENT

The authors have effectively demonstrated the potential efficacy of a novel new therapeutic modality in the management of the overactive bladder symptom complex. Clearly the results of the study need to be confirmed in appropriately powered followup studies. However, the results are encouraging in suggesting that there may be efficacy for an NK-1 antagonist supporting current concepts relating to the important potential role of the afferent system in the management of overactive bladder symptoms, thought to be related to detrusor overactivity. However, the entry criteria in this study would potentially include patients with frequency due to motor or sensory problems. This needs to be clearly defined in further followup studies. In particular, the potential efficacy of this therapy on urodynamic confirmed detrusor overactivity needs to be confirmed to support the proof of principle. Nevertheless, it is encouraging to see evidence from a human study in support of pharmacotherapy directed at afferent pathways being potentially important in the treatment of detrusor overactivity. I await the results of further studies with interest.

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